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The Effect of Leucine on the Activity of Three Enzymes in the Kynurenine Pathway of Tryptophan Metabolism in the Rat

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To the Graduate Council:

I am submitting herewith a thesis written by Barbara Elaine Blackwelder entitled "The Effect of Leucine on the Activity of Three Enzymes in the Kynurenine Pathway of Tryptophan Metabolism in the Rat." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Nutrition.

Jane R. Savage, Major Professor

We have read this thesis and recommend its acceptance:

John T. Smith, James A. Corrick

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

May 16, 1972

To the Graduate Council:

I am submitting herewith a thesis written by Barbara Elaine Blackwelder entitled "The Effect of Leucine on the Activity of Three Enzymes in the Kynurenine Pathway of Tryptophan Metabolism in the Rat." I recommend that it be accepted for nine quarter hours of credit in partial fulfillment of the requirements for the degree of Master of Science, with a major in Nutrition.

Jane R. Savage
Major Professor

We have read this thesis and recommend its acceptance:

John T. Smith

James A. Carrick, Jr.

Accepted for the Council:

Hilton A. Smith
Vice Chancellor for
Graduate Studies and Research

THE EFFECT OF LEUCINE ON THE ACTIVITY OF THREE ENZYMES IN THE KYNURENINE PATHWAY OF TRYPTOPHAN METABOLISM IN THE RAT

A Thesis

Presented to

the Graduate Council of

The University of Tennessee

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

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by

Barbara Elaine Blackwelder

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ABSTRACT

The effect of adding a mixture of indispensable amino acids simulating zein, with or without leucine, to a basal 6 percent casein, niacin-free diet on weight gain, feed consumption, and activity of kynurenine hydroxylase, 3-hydroxykynureninase, and 3-hydroxyanthranilic oxidase, three enzymes involved in the tryptophan to niacin pathway, was investigated. Three groups of Sprague-Dawley-Long Evans rats were fed one of the experimental diets for two weeks. Livers from each rat were removed and analyzed for the activity of the three enzymes under investigation.

The data obtained indicated that there were no significant differences in weight gain and feed consumption between rats fed each of the three experimental diets. In addition, the values obtained for activity of kynurenine hydroxylase also were not significantly different between rats fed each of the three diets. Although there were no significant differences in the activity of 3-hydroxykynureninase between rats fed each of the diets, the difference between the activity of 3-hydroxykynureninase in the liver of rats fed the indispensable amino acid mixture without leucine and the activity obtained from rats fed the basal diet approached significance ($P > 0.10$). The activity of 3-hydroxyanthranilic oxidase was significantly higher ($0.10 < P < 0.05$) in rats fed either of the diets containing the amino acid mixtures than in rats fed the basal diet. The activity of 3-hydroxyanthranilic oxidase

in the liver of rats fed the diet containing the amino acid mixture without leucine was significantly lower ($P < 0.05$) than the activity in rats fed the indispensable amino acid mixture containing leucine.

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CHAPTER I

INTRODUCTION

Protein malnutrition is a serious worldwide problem encountered most often in population groups consuming high amounts of staple foods such as rice, wheat, and corn. Because such foods provide poor quality protein, limiting in one or more of the essential amino acids, the cause of protein malnutrition has most commonly been considered to be due to a deficiency of indispensable amino acids. Few investigators, however, have considered the possibility that this problem also may be caused by an excess of indispensable amino acids.

Corn is a staple food containing an incomplete protein, zein, whose limiting amino acids are tryptophan and lysine. For many years the niacin deficiency disease, pellagra, has been related to population groups consuming low-protein diets in which corn contributes the major portion of the protein. Pellagra also has been observed in areas of the world where the staple food is the millet jowar. An explanation for the relationship between corn and pellagra has been the low tryptophan content of corn, since tryptophan can be converted to niacin in the body. However, the tryptophan content of jowar is not low, but is similar to that of rice and wheat, staple foods whose high consumption has not been related to pellagra.

Both corn and the millet jowar, however, possess one common feature. Both contain the indispensable amino acid leucine in relatively

high amounts. It has been suggested that the pellagragenic properties attributed to corn and jowar may be due to an amino acid imbalance resulting from their high content of leucine. In this laboratory, leucine in combination with low-protein diets has been demonstrated to interfere with the tryptophan to niacin pathway, resulting in an increased excretion of various intermediate metabolites. This study was undertaken to examine the effect of leucine on the activity of three enzymes in this pathway.

CHAPTER II

REVIEW OF THE LITERATURE

History of Pellagra

Pellagra is a serious endemic disease characterized by gastro-intestinal disturbances, neurological symptoms, and symmetrical lesions on the arms, legs, and neck. The disease may be diagnosed in the advanced stages by the classical symptoms commonly termed as the "three D's"--diarrhea, dermatitis, and dementia (1).

As early as 1735, the disease was so serious in Spain that it was described by Casal as "scorbutic leprosy" (2). He noted that its victims subsisted on low-protein diets and corn products and that the disease appeared frequently in the spring (3). Corn was the staple food of the poor in Europe because it was inexpensive and grew well in most areas. The word "pellagra" was derived by Frapolli of Italy in 1771 from "pelle" and "agra," meaning "rough skin," a term which he said was in use among the people. By the early 1900's, pellagra had become prevalent in Italy, Spain, Egypt, and Rumania (4).

When it became evident at the beginning of the twentieth century that pellagra was a serious problem in the southern United States, many theories were advanced for its cause. Possible causes were believed to be toxins, infections, food deficiencies of quantity and quality and vitamin deficiencies (2). Many observers considered corn to be a

possible factor, and in 1912 Osborne and Mendel (5) suggested that the amino acid deficiency in zein diets was the cause of pellagra.

In 1914, pellagra had reached such proportions in institutions in the South that the U.S. Public Health Service assigned Dr. Joseph Goldberger the task of investigating the cause of pellagra (6). In a paper published less than three months after beginning his investigations, Goldberger stated that the cause of the disease was dietary and that the prevention consisted of a reduction in the consumption of cereals, vegetables, and canned foods and an increase in the animal food component of the diet. He insisted that the disease was not communicable, since no cases of pellagra were reported among nurses or attendants in institutions where it was prevalent (7). Goldberger later proved the nontransmissibility of the disease after subjecting himself, his associates, and his wife to the urine, feces, blood, skin lesion scales, and nasal secretions of pellagrins (8).

Discovery of the Pellagra-Preventative Factor

Goldberger and his co-workers demonstrated in 1915 that pellagra could be prevented by a diet containing generous amounts of milk, eggs, meat, beans, and peas (9). Because of the marked pellagra-preventative value of animal protein foods and the improvement of pellagrins given tryptophan, Goldberger considered amino acid deficiency as the primary etiological factor in pellagra. In further work using soybean and casein-supplemented diets, he found that not all proteins were effective in curing the disease (6). It was finally discovered that a dried yeast

supplement, although poor in protein, prevented the disease (9). Goldberger and Tanner (10) concluded that the prevention of pellagra depended on a heretofore unknown dietary factory which they designated as factor P-P.

The pellagra-preventative factor (P-P) in foods was not isolated until the mid-1930's. In 1935, it was shown (11) that nicotinic acid was a component of coenzyme II (known today as NADP) and suggested that the substance was of metabolic importance. Nicotinic acid and its amide, nicotinamide, were isolated from liver extract by Elvejhem and co-workers (12) in 1937. These compounds proved effective in curing canine blacktongue, a disease comparable to human pellagra. In addition, nicotinic acid and nicotinamide were demonstrated to be equally effective in treating human pellagra (13, 14).

Relation of Tryptophan to Nicotinamide

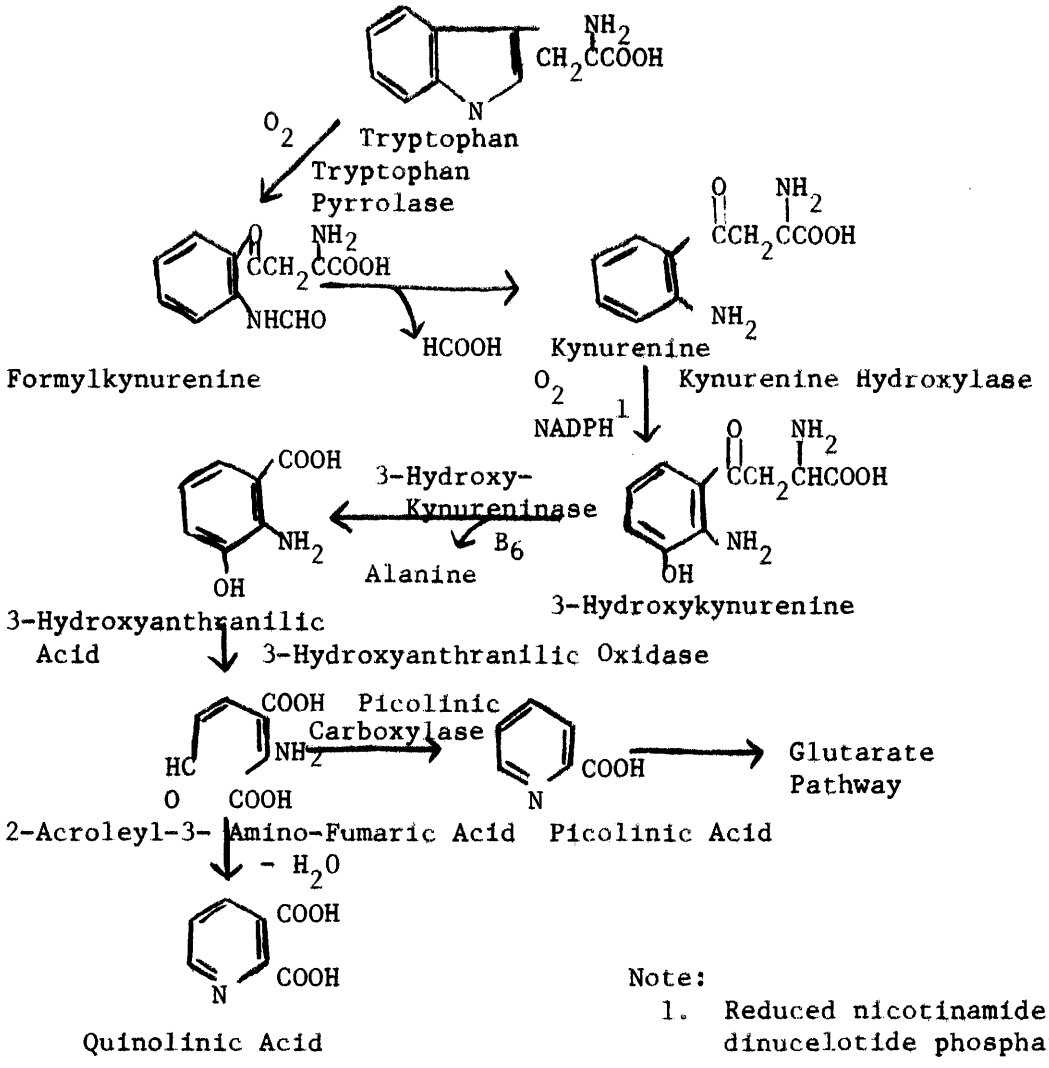
Although the discovery of nicotinamide as the pellagra-preventative factor solved the clinical problem of pellagra, many questions regarding the disease were unanswered. It was puzzling that pellagra was endemic in corn-eating populations, but not in groups consuming less nicotinic acid, nut no corn. It was also strange that milk, which was low in nicotinic acid, could cure or prevent pellagra (11). In 1945 these questions were answered when Krehl *et al.* (15) showed that rats could synthesize nicotinamide from the amino acid precursor tryptophan. In 1946, Heidelberger *et al.* (16) confirmed the fact that tryptophan is converted to metabolically active forms of nicotinic acid (NAD and NADP).

After rats were injected with DL-tryptophan in which the indole 3-carbon atom was radioactive, these investigators found the labeled atom in the carboxyl carbon of nicotinic acid, isolated from the urine of rats. Numerous intermediates were isolated and identified in further work (17, 18). Figures 1 and 2 show the series of reactions for the conversion of tryptophan to NAD and NADP. In 1956 it was demonstrated that approximately 60 mg of tryptophan are required for conversion to 1 mg of nicotinamide in humans (19).

Tryptophan Metabolism

There are two pathways by which the indispensable amino acid tryptophan may be metabolized. The most prominent route leads to the formation of two phosphopyridine nucleotide coenzymes, nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) through the series of reactions shown in Figures 1 and 2 (20). These two pyridine nucleotides, the metabolically active forms of nicotinamide, function as hydrogen carriers for a large number of dehydrogenases, especially those involved in the metabolism of carbohydrates, amino acids, and fats (21). Tryptophan is also the precursor of serotonin, which is found in many tissues, but most prominently in the intestine, brain, and platelets (Figure 3). Normally only 1 to 2 percent of the utilized tryptophan results in the formation of serotonin (20).

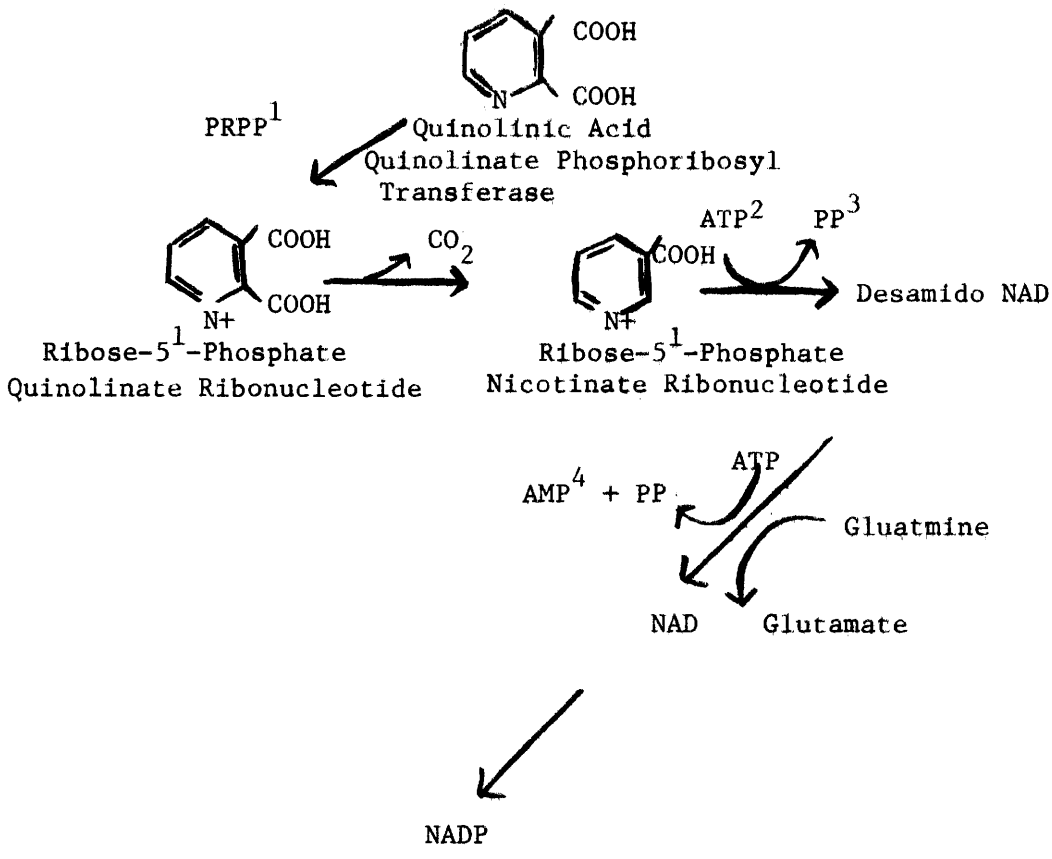
Three of the intermediates produced in the kynurenine pathway are diazotizable amines. These amines, kynurenine, 3-hydroxykynurenine, and 3-hydroxyanthranilic acid are excreted in the urine and can be



Note:

1. Reduced nicotinamide adenine dinucelotide phosphate.

Figure 1. Conversion of tryptophan to quinolinic acid.



Note:

1. 5-Phosphoribosyl-1-Pyrophosphate
2. Adenosine Triphosphate
3. Pyrophosphate
4. Adenosine Monophosphate
5. Desamido-NAD-Pyrophosphorylase

Figure 2. Conversion of quinolinic acid to NAD and NADP.

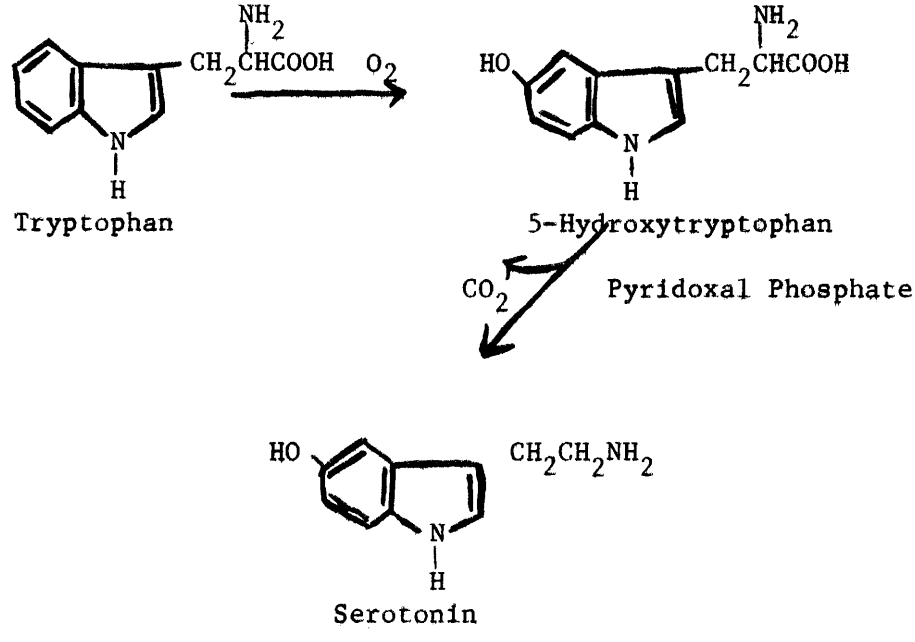


Figure 3. Serotonin pathway of tryptophan metabolism.

separated from the other metabolites for quantitative measurement. In the past decade, research on these urinary metabolites has led to numerous studies of the activity of the enzymes involved in the kynurenine pathway in various physiological conditions. These studies have been of interest because in certain conditions, such as amino acid imbalance, pyridoxine deficiency, or diabetes, a comparison of the enzymatic activity patterns can be made with the tryptophan metabolites in the urine (22).

The conversion of kynurenine to 3-hydroxykynurenine is catalyzed by the enzyme kynurenine hydroxylase. This hydroxylation, which occurs in the presence of molecular oxygen, also requires nicotinamide adenine dinucleotide phosphate (NADPH) as a coenzyme (23, 24). Kynurenine hydroxylase is localized exclusively in the outer membrane of rat liver mitochondria (25) and is independent of the cytochrome b_5 reductase system that is also localized in the outer membrane fraction. Kynurenine hydroxylase has also been found in the mitochondria of cat kidney and liver (26) and has been partially purified from the mitochondria of rat liver (27). The vitamin riboflavin is necessary for activity of this enzyme (28). Stevens and Henderson (29) demonstrated that the activity of kynurenine hydroxylase was decreased by 50 to 70 percent in the mitochondria of the liver of riboflavin-deficient rats, even after the in vitro addition of riboflavin phosphate or of flavin adenine dinucleotide from boiled liver extract.

The enzyme kynureninase catalyzes the conversion of kynurenine to anthranilic acid, while the enzyme 3-hydroxykynureninase results in

the formation of 3-hydroxyanthranilic acid from 3-hydroxykynurenine. Although kynureninase has been the more investigated of the two enzymes, researchers are not yet certain whether kynureninase and 3-hydroxy-kynureninase are two distinct enzymes (22). Kynureninase has been found in the liver of rats, guinea pigs, oxen, pigs (22), cats (26), and humans (22), in the kidney of rats and cats (26), in Pseudomonas fluorescens (30), and in Neurospora crassa (31). It has been partially purified from the supernatant fluid of rat liver homogenate (22), from Pseudomonas fluorescens (32), and from Neurospora crassa (31). Pyridoxal phosphate functions as a coenzyme for kynureninase (33). Several studies have shown that the kynureninase activity was greatly reduced in the liver of pyridoxine-deficient animals (33, 34) and could be restored in vitro by the addition of pyridoxal phosphate (33).

The enzyme 3-hydroxyanthranilic oxidase acts on 3-hydroxy-anthranilic acid to form an intermediate (amino-acroleyl-fumaric acid) from which quinolinic acid, picolinic acid, and nicotinic acid are derived (35, 36). This enzyme, which acts in the presence of ferrous and of sulfhydryl groups, has been partially purified from ox liver (35, 36, 37), and has been found in the kidney and liver of the rat and pig (38). This enzyme is almost absent in the liver of rats five days before birth, but matures rapidly in the first hours of extrauterine life. Its value is about one-half that in the adult rat liver at the moment of birth (22). Decreased activity of this enzyme has been observed in the rat liver in alloxan diabetes (39).

Staple Foods and Pellagra

As mentioned previously, pellagra has been known for many years as a classical nutritional deficiency disease affecting poor population groups who subsided on diets high in corn. In several studies in the mid-1940's, Krehl and co-workers (15, 40, 41) showed that the growth rate of rats could be depressed and symptoms suggestive of pellagra could be produced by the addition of corn to low-protein, niacin-deficient diets. This growth depression could be alleviated by the addition of either tryptophan or nicotinic acid. Corn was unique in producing this growth depression since the addition of polished rice or rolled oats to low-protein, niacin-deficient diets failed to produce these symptoms, although the latter cereals contained less nicotinic acid than corn (41, 42).

In later work, Goldsmith et al. (43) found that clinical symptoms of niacin-deficiency could be produced sooner in human subjects fed diets containing corn than in subjects fed diets containing wheat, even though the niacin and tryptophan content of the corn and wheat diets were comparable. Because this suggested that there was some constituent in corn which interfered with the utilization of tryptophan, the following reasons were theorized by these investigators to explain why pellagra symptoms appeared more rapidly in subjects fed the corn diets: (1) corn contains little tryptophan, (2) the niacin in corn may be in a bound nonutilizable form, (3) large amounts of corn may cause an amino acid imbalance, or (4) there may be a toxic substance in corn.

Pellagra is rarely observed in regions of the world where the staple foods are rice and wheat. However, it is a common disease in the Deccan Plateau of India, where it accounts for 1 percent of all general hospital admissions, and nearly 8 to 10 percent of admissions to mental hospitals in Hyderabad, India. The staple food in this region is not corn, but the millet jowar (44). This high incidence suggested to researchers that both corn and jowar could have a common feature in the etiology of pellagra. The possibility that jowar could produce canine blacktongue was investigated by Belavady et al. (45) in 1965. One group of dogs was fed a corn diet, while a second group of dogs was fed a diet containing 65 percent jowar. It was observed that classical symptoms of canine blacktongue developed in all dogs in both groups and that these symptoms disappeared upon the dietary administration of nicotinic acid. Similarly, the results of another study in 1968 indicated that diets containing corn or jowar produced pellagra in monkeys (46).

The previously stated theory which proposed that the niacin in corn is in a bound form was investigated in several studies. Pearson et al. (47) found that lime-treated maize, when added to a 9 percent casein, niacin-free diet, permitted more rapid growth of rats than did raw maize. Similarly, Kodicek (48) and Carpenter et al. (49) found that alkali treatment of maize increased the availability of nicotinic acid to the rat. However, when a comparable treatment was applied to the millet jowar, no increase in available nicotinic acid was observed (50), thus indicating that this theory could not be considered a common factor in the production of pellagra by corn and jowar.

In addition, the previously stated theory which proposed that there may be a toxic substance in corn was discounted by Horwitt (51). This investigator stated that since very large percentages of corn in diets are less pellagragenic than diets with half as much corn, there was little justification for the practical consideration of a "toxic factor" in corn.

Since pellagra is common in regions of the world where corn and jowar are staple foods and not in regions of rice and wheat consumption, the possibility was considered that these two staples may have a common feature in their chemical composition which facilitates the development of pellagra. A comparison of selected nutrients in rice, cornmeal, jowar, and wheat is reported in Table 1. As shown, these four staples are similar in carbohydrate, protein, fat, and nicotinic acid content; and although the tryptophan content of jowar varies, some strains contain amounts equal to that of rice and wheat. Corn and jowar, however, possess one common feature not observed in rice and wheat. Both staples contain the indispensable amino acid leucine in high levels. In comparison with the other staples, the leucine to tryptophan ratio shows that corn contains approximately three times as much leucine per gram of tryptophan than do either rice or wheat; and the value for jowar is almost doubled in comparison with rice or wheat.

Effects of High Leucine Diets

Numerous studies have been undertaken to investigate the relationship between leucine and pellagra. In 1960, Gopalan and

TABLE 1

A COMPARISON OF SELECTED NUTRIENTS IN RICE, CORNMEAL, JOWAR, AND WHEAT

Staple	Protein[a]	Carbo-hydrate[a]	Fat[a]	Nicotinic Acid	Tryptophan	Leucine	Leucine to Tryptophan Ratio
	g/100 g	g/100 g	g/100 g	mg/100 g	g/100 g Protein	g/100 g Protein	g/g
Rice white, raw	6.7	80.4	0.4	1.2[b]	1.2[b]	8.0[b]	6.7
Cornmeal degermed, dry	7.9	78.4	1.2	1.4[b]	0.8[b]	14.9[b]	18.6
Jowar sorghum grain	11.0	73.0	3.3	1.8[b]	1.2[b]	12.9[b]	10.8
Wheat flour, white	10.5	76.1	1.0	1.4[a]	1.2[c]	7.7[c]	6.4

[a]Watt, B. K., and A. L. Merrill 1963 Composition of Foods. Handbook No. 8, U.S. Dept. Agr., Washington, D.C., pp. 28, 52, and 57.

[b]Gopalan, C., and S. G. Srikantia 1960 Leucine and Pellagra. Lancet, 1 : 954.

[c]Orr, M. L., and B. K. Watt 1957 Amino Acid Content of Foods. Home Econ. Res. Rep. No. 4, U.S. Dept. Agr., Washington, D.C., p. 58.

Srikantia (52) fed 20 to 30 g of L-leucine to human subjects daily for seven days. They reported an increase in the urinary excretion of N^1-methylnicotinamide which could be reversed by discontinuing leucine or by supplementing the subjects' diet with nicotinic acid. They suggested that nicotinic acid depletion could occur in the tissues as a result of the amino acid imbalance caused by leucine. But in a similar study in which 4 or 10 g of L-leucine were added to adequate diets, Truswell et al. (53) did not observe any change in the urinary excretion of N^1-methylnicotinamide.

Although some researchers have suggested that leucine can influence the plasma levels of other amino acids (54), excesses of it have not been shown to affect overall nitrogen absorption, nitrogen retention, or rate of gastric emptying in rats (55).

In 1967, Rogers and co-workers (56) studied the effect of adding excesses of leucine to low-protein diets in rats. They found that leucine addition resulted in growth depression, whereas the addition of isoleucine and valine, amino acids similar in structure to leucine, largely overcame these effects. The growth rate of the rats, however, was completely restored to that of the control group only after the addition of tryptophan, phenylalanine, and threonine. Spolter and Harper (57) observed that rats adapted to high leucine diets lost their ability to grow rapidly when fed the control diet for one day, indicating that the growth retarding effects of leucine were additive. A study in 1963 indicated that the rate of utilization of isoleucine and valine by various tissues was altered when excess leucine was added to a 9 percent

casein diet (58). A later study, however, suggested that excesses of leucine involve more complex amino acid relationships than the leucine, isoleucine, and valine antagonism theory (56).

Belavady et al. (59) studied the effects of oral administration of leucine on the tryptophan to niacin pathway. An 8 percent protein diet was fed to a group of six pellagrins and six controls, after which time 10 g of L-leucine was added daily to their diet for five days. In both groups a significant increase in the excretion of N^1-methylnicotinamide was observed. In addition, there was a significant decrease in the excretion of tryptophan in the group of pellagrins. If leucine was removed from the diet, these effects were reversed in both groups. When a similar treatment of subjects with lysine produced no changes in the excretion pattern, these effects were attributed solely to leucine.

Raghuramulu et al. (60) studied the effect of adding excesses of leucine to a 9 percent casein diet in young and adult rats. When leucine was present in the diet at a 1.5 percent level, a significant increase in the excretion of N^1-methylnicotinamide was observed in adult rats. This increased excretion also occurred in young rats, but it was less significant. In addition, quinolinic acid excretion in the urine of young and adult rats was increased significantly by the presence of leucine. The addition of isoleucine to the diet counteracted the increased excretion of N^1-methylnicotinamide and quinolinic acid. These authors suggested that the increased excretion of the foregoing metabolites could be due to a block or inhibition in the conversion of quinolinic acid to niacin ribonucleotide, caused by leucine.

In an attempt to determine the basic abnormality in metabolism in pellagra, Hankes et al. (61) studied a group of twenty-two South African Bantu pellagrins who had obtained the disease from eating corn. Subjects were fed their customary maize diet and for a period of four days were given orally a dose of 2 g of L-tryptophan or 400 mg of L-kynurenine sulfate plus 100 mg of pyridoxine. Twenty-four hour urine samples were analyzed for various tryptophan metabolites. These investigators found elevated urinary levels of two or more tryptophan and niacin metabolites in 16 of the 22 subjects given an oral load of 2.0 g L-tryptophan. Kynurenine, 3-hydroxykynurenin, kynurenic acid, and xanthurenic acid were the most commonly elevated metabolites. Quinolinic acid levels in the urine were increased significantly by the administration of L-kynurenine or L-tryptophan. These authors suggested that leucine inhibited the formation of NADP, resulting in increased urinary levels of quinolinic acid. They also suggested that in pellagrins there was a lack of feedback control by NADP on the enzyme tryptophan pyrrolase.

Rao (62) observed that the addition of 3 percent L-leucine to a 9 percent casein diet resulted in a significant increase in the activity of tryptophan pyrrolase and kidney picolinic carboxylase, and a significant decrease in the activity of quinolinate phosphoribosyl transferase in the kidney and liver of rats. He suggested that the decrease in quinolinate phosphoribosyl transferase activity, brought about by leucine could result in a lowered NADP level because it was the enzyme which catalyzes the conversion of quinolinic acid to nicotinamide.

The effect of excess leucine seems to be lessened with an increase in dietary protein. A study in 1945 showed that when a 26.1 percent casein, niacin-free diet was fed to young pigs, no signs of nutritional deficiency developed except for a slightly lowered growth rate (63). Definite signs of nicotinic acid deficiency developed, however, when the protein level was lowered to 10 percent. Similarly, in 1956, Elvejhem (64) observed growth retardation in rats when a leucine supplement was added to a 9 percent casein, niacin-free diet. But no growth depression was observed when the casein level was increased to 18 percent. In 1967, Belavady et al. (65) demonstrated that signs of canine black-tongue developed within 31 to 97 days after dogs were fed a 21 percent casein, niacin-free diet, supplemented with leucine. They found, however, that dogs thrive on a 40 percent casein, 4 percent leucine diet. Therefore, they suggested that when there is a disproportionate excess of leucine over other amino acids in the diet and when the nicotinic acid level is suboptimal, changes are more likely to appear.

The effect of excess leucine on the rate of synthesis and the concentration of nicotinamide nucleotides has been studied by several investigators. In 1965, Raghuramulu et al. (66) showed that the feeding of excess leucine significantly lowered the rate of synthesis in vitro of nicotinamide nucleotides in the erythrocytes of both a normal group and a group of pellagrins. In 1964, Coulter (67) studied the effect of adding corn, zein, the complete mixture of indispensable amino acids simulating zein, with and without leucine, and a mixture of the dispensable amino acids simulating zein on the utilization of

L-tryptophan for pyridine nucleotide synthesis. Rats fed diets in which leucine was omitted from the indispensable amino acid mixture showed a significant increase in the concentration of total liver pyridine nucleotides.

Several studies at the University of Tennessee have been concerned with the effect of leucine on the urinary excretion of various metabolites in the kynurenine pathway. In 1966, Acacio (68) studied the effect of zein or a mixture of all the indispensable amino acids simulating 1.71 percent zein, with or without leucine, on the excretion of the metabolites in rats fed a 6 percent casein, niacin-free diet. Each of these diets was fed with or without a 0.1 percent tryptophan supplement and pooled 24-hour urine samples were collected and analyzed for kynurenine, 3-hydroxykynurenine, and 3-hydroxyanthranilic acid. She found that leucine, the indispensable amino acid found in zein in the largest amount, interfered with the conversion of tryptophan to 3-hydroxykynurenine. However, the results of her study were based on a single pooled urine sample from each experimental group. Thus no statistical significance could be attached to the observed differences.

In 1970, Wallin (69) tested the reliability of Acacio's results in a similar study using comparable diets. She measured the urinary excretion of the tryptophan metabolites kynurenine, 3-hydroxykynurenine, and 3-hydroxyanthranilic acid in two pooled urine samples from each group so that statistical significance of the data could be determined. Wallin found that the excretion of kynurenine in rats fed the complete amino acid mixtures with leucine was significantly higher as compared

to rats fed the amino acid mixtures without leucine; and that there was a concomitant decrease in 3-hydroxykynurenine excretion. She suggested that leucine causes a block in the tryptophan to NAD pathway between these two metabolites. She also found that the excretion of 3-hydroxyanthranilic acid was higher in rats fed the complete amino acid mixtures with leucine as compared to rats fed the amino acid mixtures without leucine. This was interpreted to mean that leucine may cause a block in the pathway past 3-hydroxyanthranilic acid, resulting in a buildup of that metabolite.

Since Wallin (69) clearly established that leucine causes at least two blocks in the tryptophan to NAD pathway, the present study was designed to determine if these blocks were due to altered enzyme activity of three enzymes in this pathway. The enzymes studied were those which catalyze the reactions that Wallin found to be blocked by leucine. These enzymes are kynurenine hydroxylase, 3-hydroxykynureninase, and 3-hydroxyanthranilic oxidase. On the basis of Wallin's work, which indicated that there is a block between kynurenine and 3-hydroxykynurenine, the activity of kynurenine hydroxylase might be lowered, if the block is due to altered enzyme activity. Similarly, a block between 3-hydroxykynurenine and 3-hydroxyanthranilic acid might result in lowered enzyme activity of 3-hydroxykynureninase. And if leucine causes a block in the pathway past 3-hydroxyanthranilic acid, the activity of 3-hydroxyanthranilic oxidase might be lowered.

CHAPTER III

EXPERIMENTAL PROCEDURE

Diets

The basal diet fed to rats in the present study was composed of (in percent) vitamin-free casein, 6; corn oil containing fat-soluble vitamins, 5; choline, 0.15; DL-methionine, 0.3; water-soluble vitamin mixture, 0.25; Hubbell, Mendel, and Wakeman salt mix (70), 3; L-tryptophan, 0.1; and sucrose, 85.2. All additions to the basal diet were made at the expense of sucrose. Tables 2 and 3 show the compositions of the fat-soluble and water-soluble vitamin mixtures, respectively.

Krehl and co-workers (40) showed that niacin deficiency could be produced in rats fed a low-protein, niacin-free ration containing 40 percent corn. Coulter (67) estimated that 40 g of corn contains 1.71 percent zein and developed mixtures of the dispensable and indispensable amino acids which simulated those provided in a 1.71 percent zein diet. In the present study one group of rats was fed the basal ration described above. Another group was fed the basal ration plus all the indispensable amino acids as would be found in a diet containing 1.71 percent zein. A third group was fed the basal ration plus a similar amino acid mixture as the second group except that leucine was omitted.

TABLE 2

COMPONENTS OF THE FAT-SOLUBLE VITAMIN MIXTURE

Component	Amount[a] (grams)
Calciferol	0.006
α-DL-tocopherol	12.750
Halibut Liver Oil	8.500
Mazola Corn Oil	616.000

[a]Five grams of this mixture per hundred grams of diet were used.

TABLE 3

COMPONENTS OF THE NIACIN-FREE, WATER-SOLUBLE VITAMIN MIXTURE

Component	Amount (grams)
Thiamine HCl	0.250
Riboflavin	0.250
Calcium Panthothenate	1.000
Pyridoxine HCl	0.130
Folic Acid	0.010
Menadione	0.030
Biotin	0.005
Vitamin B_{12} (1 g triturate) 0.1 percent with mannitol	0.001
Inositol	5.000
Ascorbic Acid	2.500
Sucrose	115.824

The composition of the mixture of indispensable amino acids simulating those in a diet containing 1.71 percent zein is shown in Table 4.

Experimental Animals

Twenty-seven male weanling rats,[1] ranging in weight from 31 to 78 g, were obtained for use in this study. All animals were caged individually and fed the basal diet and distilled water ad libitum for five days to allow them to adjust to experimental conditions. At the end of five days, the rats were weighed and divided into three groups of nine rats each so that the average weights of the three groups did not differ by more than one gram.

Each group of animals was fed one of the experimental diets described above and distilled water ad libitum for two weeks. They were weighed at the beginning, middle, and end of the two-week period. Feed consumption was measured three times a week. At the end of the two-week period, the animals were sacrificed by decapitation. Immediately the liver from each rat was removed and chilled. After the liver was blotted dry, it was divided into three sections. Each section of liver was weighed using an O'Haus balance. Each sample, which weighed approximately one gram each, was used for determination of enzyme activity of kynurenine hydroxylase, 3-hydroxykynureninase, and 3-hydroxyanthranilic oxidase. The method outlined by Chiancone (22)

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[1]"Long-Evans-Sprague-Dawley"; Nutrition Department, The University of Tennessee, Knoxville, Tennessee.

TABLE 4

MIXTURE OF INDISPENSABLE AMINO ACIDS

Amino Acid	Amount[a] g/100 g Diet
L-Tryptophan	0.0015
L-Lysine (Lysine Monochloride 0.0019 g)	0.0015
L-Histidine (Histidine Monochloride 0.0243 g)	0.0180
L-Arginine (Arginine Hydrochloride 0.0293 g)	0.0240
L-Methionine	0.0320
L-Valine	0.0390
L-Threonine	0.0410
L-Phenylalanine	0.0970
L-Isoleucine	0.1100
L-Leucine	0.3300

[a]Amounts of indispensable amino acids equivalent to that provided by a diet supplying 1.71 percent zein.

was followed for these determinations, with some modifications to suit the prevailing conditions in the laboratory.

Determination of Enzyme Activity of Kynurenine Hydroxylase

The enzyme kynurenine hydroxylase catalyzes the transformation of kynurenine to 3-hydroxykynurenine. The activity determination is based on the assay of 3-hydroxykynurenine which is formed during the incubation of mitochondria with kynurenine in the presence of NADPH.

The liver samples for kynurenine hydroxylase determination, removed immediately after sacrifice and exsanguination of the animal, were chilled, blotted dry, and weighed on an O'Haus balance. The samples, weighing approximately one gram, were placed in homogenizing tubes containing nine volumes of ice-cold 0.25 M sucrose solution with 0.001 M EDTA. The samples were kept cold in an ice bath during the determination. The samples were homogenized using a Potter-Elvejhem teflon homogenizer and centrifuged[2] at 600 X g for 10 minutes at 4°C. The supernatant fluid was decanted into chilled centrifuge tubes and centrifuged at 13,000 X g for 10 minutes at 4°C. The sediment (mitochondria) was washed by suspending it by homogenization in a volume of ice-cold 0.25 M sucrose-EDTA solution nine times the volume of tissue used for the preparation, and was centrifuged at 20,000 X g for 10 minutes at 4°C. The washing was repeated a second time. The supernatant fluid was decanted and the mitochondria were suspended in a volume of ice-cold

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[2]"Beta-Fuge," Model A, Lourdes Instrument Corporation, Brooklyn, New York.

0.25 M sucrose solution corresponding to one-half that of the original tissue. An equal volume of an ice-cold 2 percent sodium cholate solution at pH 7.8 was added. The mitochondrial suspension was left at 2°C for 90 minutes, being stirred often with a glass rod. A portion of the mixture was transferred to another tube, capped, frozen, and saved for nitrogen determination by the micro-Kjeldahl method to be described later. At the end of the 90-minute period, a 0.3 ml aliquot of the liver mitochondrial suspension was transferred to a plastic centrifuge tube. To it, the following reagents were added: 0.5 ml of 0.3 M tris buffer, pH 8.3; 0.3 ml of 0.1 M KCl; 0.3 ml of 0.2 M nicotinamide; 0.3 ml of 0.1 M KCN; 0.1 ml of 0.05 M cysteine; 0.1 ml of 0.01 M L-kynurenine sulfate; and 1.0 ml of twice-distilled water. Another tube was prepared containing all the reagents listed above except no liver mitochondrial suspension was added. This mixture served as a blank. Both sample and blank mixtures were incubated[3] with shaking at 30°C for five minutes. At the end of five minutes, 0.1 ml of 0.005 M NADPH was added with a pipette to both mixtures; and immediately after the addition of NADPH, 1.0 ml of 16 percent trichloroacetic acid was added to the blank. Both sample and blank mixtures were incubated at 30°C for 10 minutes, after which the sample mixture was deproteinized by the addition of 1.0 ml of 16 percent trichloroacetic acid. Both mixtures were centrifuged[4] at 300 X g for 10 minutes. The supernatant

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[3]"Water-Bath Shaker," Eberbach Corp., Ann-Arbor, Michigan.

[4]"International Centrifuge," Model SBV, Boston, Massachusetts.

fluid was decanted into a test tube and used for measurement of 3-hydroxykynurenine according to the spectrophotometric method with nitrous acid as follows. One milliliter of the trichloroacetic acid filtrate (supernatant) was transferred to a small, chilled test tube and the following reagents were added: 0.1 ml of twice-distilled water; 1.0 ml of 1 N HCl; and 0.2 ml of 0.25 percent freshly prepared sodium nitrite solution. Another tube was prepared containing all the reagents listed above except 0.2 ml of twice-distilled water was substituted for sodium nitrite solution. This tube served as a blank. In order for the colorimetric reaction to take place, the tubes were kept dark by being placed under a black cloth and were shaken frequently. Three minutes after the addition of sodium nitrite, 0.2 ml of 10 percent ammonium sulfamate solution was added to each tube, and the mixture was shaken thoroughly to eliminate excess nitrogen. Ten minutes after the addition of sodium nitrite, the optical density was determined against a reagent blank using the Beckman model B spectrophotometer at 390 mμ. The instrument was zeroed using distilled water. The enzymatic activity was expressed as the increase in micromoles of 3-hydroxykynurenine formed in one hour by one milligram of mitochondrial protein, by applying the following formula:

$$\mu\text{moles of 3-hydroxykynurenine/h/mg protein} = (\Delta\ \text{O.D.})\ (K)\ X\ \frac{4\ X\ 6\ X\ 3.3}{\text{mg protein in 1 ml mitochondrial suspension}}$$

where:

Δ O.D. = optical density of sample minus optical density of blank,

K = constant value of 0.768,

4 = total volume (in milliliters) of the deproteinized mixture,

6 = factor for adjusting the 10 minute incubation period to 1 hour of incubation, and

3.3 = factor for adjusting the 0.3 ml mitochondrial suspension to 1 ml of mitochondrial suspension.

Determination of Enzyme Activity of 3-Hydroxykynureninase

The enzyme 3-hydroxykynureninase catalyzes the transformation of 3-hydroxykynurenine to 3-hydroxyanthranilic acid. The determination of 3-hydroxykynureninase activity is made by determining the decrease per unit time of the concentration of 3-hydroxykynurenine in an incubation mixture containing liver homogenate.

The liver samples for 3-hydroxykynureninase determination, removed immediately after sacrifice and exsanguination of the animal, were chilled, blotted dry, and weighed on O'Haus balance. The samples, weighing approximately one gram, were placed in homogenizing tubes containing two volumes of ice-cold 0.14 M KCl solution. The samples were kept cold in an ice bath during the determination. The samples were homogenized using a Potter-Elvejhem teflon homogenizer and centrifuged[5]

[5]"Lourdes Centrifuge," Harshaw Chemical Company, Cleveland, Ohio.

at 600 X g for five minutes at 4°C. The supernatant fluid was decanted into dialyzing bags, and dialyzed for two hours against ice-cold distilled water at 2°C. The water was changed frequently. A portion of this mixture was transferred to another tube, capped, frozen, and saved for nitrogen determination by the micro-Kyeldahl method. After the samples had dialyzed, a 0.5 ml aliquot of the homogenate, corresponding to approximately 160 mg of wet tissue, was transferred to a plastic centrifuge tube and stoppered. To it, the following reagents were added: 0.5 ml of 0.2 M phosphate buffer, pH 8; and 0.2 ml of 0.0075 M L-3-hydroxykynurenine. Another tube was prepared containing all of the reagents listed above, except no L-3-hydroxykynurenine was added. This mixture served as a blank. Both sample and blank mixtures were incubated for 40 minutes at 38°C. At the end of 40 minutes, the sample mixture was deproteinized by the addition of 7.0 ml of 1 percent boric acid in absolute ethanol. Seven milliliters of 1 percent boric acid in absolute ethanol also were added to the blank. Immediately after incubation 0.2 ml of L-3-hydroxykynurenine was added to the blank. The sample and blank mixtures were centrifuged[6] at 300 X g for 10 minutes, and the supernatant fluid was decanted into small test tubes. The optical density of the supernatant fluid was determined against a reagent blank using the Beckman model B spectrophotometer at 378 mμ. The instrument was zeroed using distilled water. The enzymatic activity was expressed

[6]"International Centrifuge," Model SBV, Boston, Massachusetts.

in terms of the decrease in micromoles of 3-hydroxykynurenine in one hour by an amount of homogenate containing 100 mg of total nitrogen, by applying the following formula:

$$\mu\text{moles of 3-hydroxykynurenine/h/100 mg nitrogen} = (\Delta\ \text{O.D.})\ (K)\ X\ \frac{8.2\ X\ 2\ X\ 1.5}{\text{mg nitrogen in 1 ml homogenate}}$$

where:

Δ O.D. = optical density of blank minus optical density of sample,

K = constant value of 0.308, which represents the molar extinction coefficient of 3-hydroxykynurenine (22),

8.2 = total volume (in milliliters) of the deproteinized incubation mixture,

2 = factor for adjusting the 0.5 ml of homogenate to 1 ml of homogenate, and

1.5 = factor for adjusting the 40 minute incubation to 1 hour of incubation.

Determination of Enzyme Activity of 3-Hydroxyanthranilic Oxidase

The enzyme 3-hydroxyanthranilic oxidase catalyzes the oxidation of 3-hydroxyanthranilic acid to amino-acroleyl fumaric acid (2-acroleyl-3-amino fumaric acid). The determination of 3-hydroxyanthanilic oxidase activity is carried out by evaluating the increase of amino-acroleyl fumaric acid in a mixture containing 3-hydroxyanthranilic acid as substrate.

The liver samples for 3-hydroxyanthranilic oxidase determination, removed immediately after sacrifice and exsanguination of the animal, were chilled, blotted dry, and weighed on an O'Haus balance. The samples, weighing approximately one gram, were placed in homogenizing tubes containing two volumes of ice-cold 0.14 M KCl solution. The samples were kept cold in an ice bath during the determination. The samples were homogenized using a Potter-Elvejhem teflon homogenizer and centrifuged[7] at 18,000 X g for 10 minutes at 4°C. The supernatant fluid was decanted into small test tubes. A 1:10 dilution of the homogenate was made by adding 0.5 ml of the supernatant fluid to 4.5 ml of 0.14 M KCl in a test tube. A portion of the 1:10 diluted sample was transferred to another tube, capped, frozen, and saved for nitrogen determination by the micro-Kjeldahl method. One-hundred microliters of the diluted supernatant fluid, corresponding to approximately 3.3 mg of wet tissue, were transferred to a small test tube with a micro-pipette. The following reagents were also added to the tube: 1.0 ml of 0.1 M phosphate buffer, pH 7.5, and 5.0 ml of twice-distilled water. The optical density of the mixture was determined using the Beckman model B spectrophotometer at 360 mμ. The instrument was zeroed with a reagent blank. Optical density readings of the mixture were taken at 15- to 20-second intervals until constant values were obtained. After this occurred, 0.2 ml of 0.7 mM of 3-hydroxyanthranilic acid was added to the mixture. A piece of parafilm was placed over the top

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[7] "Lourdes Centrifuge," Harshaw Chemical Corporation, Cleveland, Ohio.

of each tube and the tubes were inverted two times. After 3-hydroxyanthranilic acid was added, optical density readings were taken at 60-second intervals for two minutes. The activity of 3-hydroxyanthranilic oxidase was expressed as the difference between the optical densities obtained immediately before and 60 seconds after the addition of 3-hydroxyanthranilic acid. The following formula was used to calculate enzyme activity:

$$\mu\text{moles of amino-acroleyl fumaric acid/min/mg nitrogen} = \frac{\Delta \text{ O.D./100 } \mu\text{l X 10}}{\text{mg total nitrogen in 1 ml homogenate}},$$

where:

10 = factor for adjusting the 100 µl of homogenate to 1 ml of homogenate.

Determination of Nitrogen by the Micro-Kjeldahl Method

The method outlined by Willits and Ogg (71) was used for this determination. The samples used were the liver mitochondrial suspension for kynurenine hydroxylase; the dialyzed supernatant fluid for 3-hydroxykynureninase; and the 1:10 diluted supernatant fluid for 3-hydroxyanthranilic oxidase, all of which were described above. Nitrogen determination of each sample was done in duplicate. One-half milliliter of each sample was pipetted into a 30 ml Kjeldahl digestion flask. To each sample, the following reagents were added: 1.3 ± 0.05 g of sodium sulfate; 40 ± 5 mg of mercuric oxide; and

2.0 ml of concentrated sulfuric acid. Another flask was prepared containing all the reagents listed above except no sample was added. This flask served as a blank. The samples and blanks were digested by boiling vigorously for a minimum of four hours. The digestion was judged to be complete when the contents of the flasks turned clear and there was no trace of black specks. The samples were cooled, and 5.0 ml of distilled water was added to each flask to dissolve the white solids. The samples were quantitatively transferred to a preheated, micro-steam distillation apparatus. Transfer of nitrogen from digestion flask was checked by adding a drop of methyl orange indicator. A pink color indicated that nitrogen was present, while an orange color indicated the absence of nitrogen. Eight milliliters of a sodium-hydroxide-sodium-thiosulfate solution were added to each sample. The samples were steam-distilled until approximately 14 ml of distillate were collected in a 125 ml Erlenmeyer flask containing 5 ml of 4 percent boric acid solution with four drops of methyl-red-bromcresol-green indicator. The contents of the flasks were diluted to 50 ml with distilled water, and were titrated with 0.01 N HCl. The first appearance of a clear color was judged to be the end point of the titration. Nitrogen was calculated by the following formula:

$$\text{Grams nitrogen per milliliter} = \frac{(\text{ml of HCl} - \text{blank})(\text{N of HCl})(\text{Meq Wt Nitrogen})}{\text{Volume of Homogenate}}$$

For kynurenine hydroxylase, protein was calculated from grams of nitrogen using the factor of 6.25 g protein per gram of nitrogen.

Statistical Analysis

The mean weight gains and feed consumption of the rats per 14 days of the experiment were calculated. The standard errors of these means were then determined. A least squares analysis of variance as described by Harvey (72) was calculated to determine differences in weight gain and feed consumption between rats fed the various experimental diets.

The mean enzyme activity and standard error of the mean for the activity of the three enzymes also were calculated. Student's "t" test for nonpaired experiments (73) was used to determine significance of differences observed in the activity of the enzymes between rats fed the various experimental diets.

CHAPTER IV

RESULTS AND DISCUSSION

Weight Gain and Feed Consumption

The effects of the various dietary treatments on weight gain and feed consumption of rats are reported in Table 5. Rats fed the basal diet (Group A), the basal diet plus the indispensable amino acid mixture containing leucine (Group B), and the basal diet plus the indispensable amino acid mixture without leucine (Group C) had weight gains of 16, 19, and 19 g per 14 days, respectively. And as shown, mean values for feed consumption were 105, 112, and 123 g per 14 days for Group A, Group B, and Group C, respectively. Weight gains and feed consumption were not significantly different between rats fed the various diets.

The growth rate of rats fed the basal diet in the present study was consistent with that observed by Acacio (68), who reported an average weight gain of 19 g per 14 days, using a similar basal diet as that used in the present study. However, the observed growth rate in the present study did not agree with results reported by Griffin (74) and Wallin (69), who also used comparable basal diets. Griffin reported an average weight gain of 7 g per 14 days, whereas Wallin reported a considerably lower average weight gain of 0.6 g per 14 days. However, half of the ten rats fed the basal diet in Wallin's study lost weight. She reported that a weight gain of 5 g per 14 days would have

TABLE 5

THE EFFECT OF A MIXTURE OF INDISPENSABLE AMINO ACIDS AS FOUND IN ZEIN, WITH OR WITHOUT LEUCINE, ON THE WEIGHT GAIN AND FEED CONSUMPTION OF RATS

Diet[a]	Number of Animals	Total Weight Gain g/14 days	Total Feed Consumed g/14 days
A	9	16 ± 1[b]	105 ± 8[b]
B	9	19 ± 2	112 ± 9
C	9	19 ± 1	123 ± 10

[a]Code for diets: "A"--basal; "B"--basal + mixture of indispensable amino acids simulating 1.71 percent zein with leucine; "C"--basal + mixture of indispensable amino acids simulating 1.71 percent zein without leucine.

[b]Mean ± standard error of the mean.

been observed if weight gain had been calculated from only the five rats that she selected for urine collection and measurement of urinary tryptophan metabolites. This value would have been more comparable to that observed by Griffin.

These differences in weight gain between the various studies cannot be explained on the basis of strain of rats used. Although Griffin (74) and Wallin (69) used Sprague-Dawley rats, they reported lower weight gains than those reported in the present study. But using Wistar rats, Acacio (68) reported weight gains comparable to those in the present study. In both the present study and in that of Acacio, the rats used were available locally, whereas the rats used by Griffin and Wallin had to be transported to the experimental facilities. Furthermore, neither Griffin nor Wallin reported placing their rats on a preexperimental adjustment period prior to feeding them the experimental diets. In the present study, after the rats were weaned, they were fed the basal diet ad libitum for five days prior to being placed on the experimental diets. This enabled the rats to adjust to experimental conditions. Therefore, it is likely that the lower weight gains of rats reported by Griffin and Wallin may be due to adverse travel conditions and lack of time for adequate adjustment to experimental conditions. Although the rats in Acacio's study also lacked a preexperimental adjustment period, they had not been subjected to stress in traveling.

Another factor that deserves mention is the composition of the basal diet used in the present study. The basal diet was identical in

composition to basal diets used by Griffin (74), Acacio (68), and Wallin (69) except for the percentage of sucrose and salt mixture (70) used. In the present study, the percentage of sucrose was increased from 83.2 percent, which had been used in earlier studies, to 85.2 percent, and the salt mixture was dropped from 5 percent to 3 percent.

In spite of the above differences in weight gain for rats fed the basal diet, the weight gains of rats fed the diets containing the indispensable amino acid mixtures were consistent with those observed in earlier studies. Acacio (68) reported an average weight gain of 21 g per 14 days for both groups of rats fed the indispensable amino acid mixtures, with or without leucine, while Wallin (69) reported somewhat lower average weight gains of 18 g and 13 g per 14 days for rats fed the indispensable amino acid mixture containing leucine and the indispensable amino acid mixture without leucine, respectively.

Rogers et al. (56) reported that rats fed a 9 percent casein diet supplemented with 5 percent leucine had significantly lower weight gains than rats fed a basal 9 percent casein diet. Therefore, one might expect a lower average weight gain in rats fed the indispensable amino acid mixture containing leucine than for rats fed the indispensable amino acid mixture without leucine or for rats fed the basal diet. But this reported lowered growth effect caused by leucine was not observed in the present study or in studies by Acacio (68) or Wallin (69). However, in the present study and in those of Acacio and of Wallin, the leucine content of the complete indispensable amino acid mixture was

only 0.3 percent leucine, which might indicate that the effect of excess dietary leucine cannot be seen at this lower level of leucine ingestion.

Enzyme Activity of Kynurenine Hydroxylase

In Table 6, mean values of 0.9, 1.2, and 0.9 μM of 3-hydroxykynurenine/h/mg protein are reported for the enzyme activity of kynurenine hydroxylase in the liver of rats fed the basal diet, the indispensable amino acid mixture containing leucine, and the indispensable amino acid mixture without leucine, respectively. These values were not significantly different between rats fed the various diets. The value for rats fed the basal diet, however, was not consistent with values reported in the literature. Using similar analytical methods, Tenconi (75) reported a mean value of 0.18 ± 0.03 μM of 3-hydroxykynurenine/h/mg protein for the activity of kynurenine hydroxylase in the livers of seven-month old rats.

Calculations for the activity of kynurenine hydroxylase in rats fed the basal diet were made from the observations obtained from all eight rats which were fed this diet. Of the eight rats fed the diet containing the complete amino acid mixture, calculations for the activity of this enzyme were made from the observations obtained from six rats. Two observations were discarded because it appeared that the enzyme had been denatured in the experimental procedure and because part of one sample was accidentally spilled, the activity of this enzyme in rats fed the amino acid mixture without leucine was calculated from observations obtained from seven rats.

TABLE 6

THE EFFECT OF A MIXTURE OF INDISPENSABLE AMINO ACIDS AS FOUND IN ZEIN, WITH OR WITHOUT LEUCINE, ON THE ENZYME ACTIVITY OF KYNURENINE HYDROXYLASE

Diet[a]	Number of Animals	Kynurenine Hydroxylase μM 3-OH-Kynurenine/h/mg Protein
A	8	0.9 ± 0.2[b]
B	6	1.2 ± 0.3
C	7	0.9 ± 0.2

[a]Code for diets: "A" -- basal; "B" -- basal + mixture of indispensable amino acids simulating 1.71 percent zein with leucine; "C" -- basal + mixture of indispensable amino acids simulating 1.71 percent zein without leucine.

[b]Mean ± standard error of the mean.

The activity observed for kynurenine hydroxylase in the present study was not consistent with the proposal of Wallin (69) who suggested that leucine causes a block in the tryptophan to NAD pathway between the metabolites kynurenine and 3-hydroxykynurenine. Wallin observed that rats fed the complete indispensable amino acid mixture excreted significantly higher ($P < 0.01$) amounts of kynurenine than rats fed the basal diet. She also observed that when leucine was removed from the indispensable amino acid mixture, rats excreted significantly lower ($P < 0.05$) amounts of kynurenine as compared to the value obtained for rats fed the diet containing the complete amino acid mixture. Since Wallin found no signficant differences in weight gain and feed consumption between the two groups fed the amino acid mixtures, she suggested that these differences in kynurenine excretion were an indication of a specific effect brought about by leucine rather than differences in nitrogen or tryptophan intake of the two groups.

On the basis of Wallin's study (69) which proposed that leucine causes a block between the metabolites kynurenine and 3-hydroxykynurenine, one might expect lowered activity of kynurenine hydroxylase, if the block is due to altered enzyme activity. However, as stated previously, no signficant differences were shown in the present study for the activity of kynurenine hydroxylase in the liver of rats fed the various diets. Because the level of leucine used in this study did not cause lowered kynurenine hydroxylase activity in rats fed the amino acid mixtures containing leucine, one might conclude that the block between

the metabolites kynurenine and 3-hydroxykynurenine is due to some unidentified factor rather than altered enzyme activity.

Enzyme Activity of 3-Hydroxykynureninase

Mean values of 6.0, 18.7, and 13.5 μM of 3-hydroxykynurenine per hour per 100 mg N are shown in Table 7 for the enzyme activity of 3-hydroxykynureninase in the liver of rats fed the basal diet, the indispensable amino acid mixture containing leucine, and the indispensable amino acid mixture without leucine, respectively. The value obtained in the present study for the activity of this enzyme in the liver of rats fed the basal diet was somewhat lower than a value reported in the literature by Ginoulhiac et al. (76). Using similar analytical methods to those used in the present study, these authors reported a value of 9.48 ± 1.12 μM 3-hydroxykynurenine/h/100 Mg N for the activity of this enzyme in rat liver. No information was available on diet or age of rats used in the study.

In the present study, there were no significant differences in the activity of 3-hydroxykynureninase between rats fed the various diets. Although not significantly different, the activity of this enzyme in the livers of rats fed both of the indispensable amino acid mixtures was higher than the activity in rats fed the basal diet. Also, the value obtained for rats fed the indispensable amino acid mixture without leucine (Group C) approached significance ($P > 0.10$) as compared to the value obtained from rats fed the basal diet (Group A).

Considerable variation was observed in the values obtained for the activity of this enzyme in rats fed each of the three diets. Of

TABLE 7

THE EFFECT OF A MIXTURE OF INDISPENSABLE AMINO ACIDS AS FOUND IN ZEIN, WITH OR WITHOUT LEUCINE, ON THE ENZYME ACTIVITY OF 3-HYDROXYKYNURENINASE

Diet[a]	Number of Animals	3-Hydroxykynureninase μM 3-OH-Kynurenine/h/100 mg N
A	6	6.0 ± 1.9[b]
B	4	18.7 ± 11.4
C	5	13.5 ± 4.9[c]

[a]Code for diets: "A" -- basal; "B" -- basal + mixture of indispensable amino acids simulating 1.71 percent zein with leucine; "C" -- basal + mixture of indispensable amino acids simulating 1.71 percent zein without leucine.

[b]Mean ± standard error of the mean.

[c]Value for rats fed Diet A was lower than value for rats fed Diet C. This difference approached significance ($P < 0.10$).

the eight rats fed each of the diets, enzyme activity was calculated from observations made in 6, 4, and 5 rats for Diets, A, B, and C, respectively. In the discarded observations, there appeared to be no difference between the optical density readings of the blank and sample, indicating inactivity of the enzyme.

Wallin (69) found no significant differences in the excretion of 3-hydroxykynurenine between rats fed the basal diet and rats fed the two amino acid mixtures. However, she observed that the mean excretion value for 3-hydroxykynurenine in rats fed the complete amino acid mixture was significantly lower ($P < 0.01$) than the value obtained from the group of rats fed the amino acid mixture without leucine.

On the basis of Wallin's study (69), which reported that the presence of leucine in the amino acid mixture caused a lowered excretion of 3-hydroxykynurenine, one might expect lowered activity of 3-hydroxykynureninase in rats fed the indispensable amino acid mixture containing leucine, if leucine adversely affects the activity of this enzyme. However, as stated previously, no significant differences were shown in the activity of this enzyme in rats fed the various diets. The high activity of this enzyme in the liver of rats fed the complete amino acid mixture (although not significantly different from the other values) and the low urinary excretion of 3-hydroxykynurenine observed by Wallin for rats fed the same diet might indicate that leucine causes an increased metabolization of 3-hydroxykynurenine. But due to inconclusive data and the high standard error of the mean for the activity of this enzyme in rats fed the indispensable amino acid

mixture containing leucine, no definite statement can be made about the effect of leucine on the activity of this enzyme.

It is difficult to determine whether the high value for the activity of 3-hydroxykynureninase in rats fed the amino acid mixture containing leucine was due to a specific effect of leucine on this enzyme or to error introduced by the experimental procedure (as was indicated by the high standard error of the mean). Of eight rats fed the diet containing the complete amino acid mixture, half were used for calculation of enzyme activity. The values obtained from the other four rats were discarded because there were no differences in the optical density readings of the blank and sample, indicating inactivity of the enzyme. The four values which were used to calculate the mean activity of this enzyme ranged in value from 1.19 to 52.41 μM 3-hydroxykynurenine per hour per 100 Mg N, resulting in the high standard error of the mean.

It is also possible that error was introduced by faulty technique in the micro-Kjeldahl procedure for the analysis of nitrogen in the dialyzed supernatant fluid used for 3-hydroxykynureninase determination. As stated previously, nitrogen determination for all samples of the liver homogenates were done in duplicate. Although for most samples, the milliliters of 0.01 N HCl required to titrate duplicates of the same sample were consistent, variability was observed between duplicates of the same sample for this enzyme in particular. In some instances, for example, there was between two to four milliliters difference in the amount of HCl required to titrate duplicates of the same sample. One possible cause for this variability was the quantity of supernatant

fluid that was available for analysis of nitrogen by the micro-Kjeldahl procedure. As stated previously, after the liver samples for 3-hydroxykynureninase determination were homogenized in two volumes of 0.14 M KCl, they were centrifuged. The supernatant fluid was poured into dialyzing bags and after dialysis, it was transferred to another tube, capped, and frozen for nitrogen determination. Because only 2 ml of 0.14 M KCl were used for homogenization, the quantity of the supernatant fluid obtained after centrifuging was obviously very small. In many samples, this small quantity of supernatant fluid appeared non-homogeneous. Unfortunately, no more sample was available to allow additional nitrogen determinations to be done.

Enzyme Activity of 3-Hydroxyanthranilic Oxidase

In Table 8, mean values of 0.7, 1.4, and 1.0 μM amino-acroleyl-fumaric acid/min/mg N are reported for the enzyme activity of 3-hydroxyanthranilic oxidase in the liver of rats fed the basal diet, the indispensable amino acid mixture containing leucine, and the indispensable amino acid mixture without leucine, respectively. The value calculated for rats fed the basal diet was lower than values reported in the literature. Using similar analytical methods to that used in the present study, Tenconi (75) reported a value of 7.64 ± 1.60 μM amino-acroleyl-fumaric acid/min/mg N in the livers of seven-month old rats.

Of the eight rats fed Diet A and of the eight rats fed Diet C, observations taken from seven rats were used to calculate the activity of this enzyme. The other observations were discarded from the calculations because it appeared that the enzyme had been denatured.

TABLE 8

THE EFFECT OF A MIXTURE OF INDISPENSABLE AMINO ACIDS AS FOUND IN ZEIN, WITH OR WITHOUT LEUCINE, ON THE ENZYME ACTIVITY OF 3-HYDROXYANTHRANILIC OXIDASE

Diet[a]	Number of Animals	3-Hydroxyanthranilic Oxidase μM amino-acroleyl-fumaric acid/min/mg N
A	7	0.7 ± 0.1[b,c]
B	8	1.4 ± 0.2[d]
C	7	1.0 ± 0.1[e]

[a]Code for diets: "A"-- basal; "B" -- basal + mixture of indispensable amino acids simulating 1.71 percent zein with leucine; "C" -- basal + mixture of indispensable amino acids simulating 1.71 percent zein without leucine.

[b]Mean ± standard error of the mean.

[c]Significantly lower than Diet B ($P < 0.05$) and Diet C ($P < 0.10$).

[d]Significantly higher than Diet C ($P < 0.10$) and Diet A ($P < 0.05$).

[e]Significantly higher than Diet A ($P < 0.05$) and lower than Diet B ($P < 0.10$).

As shown in Table 8, the enzyme activity of 3-hydroxyanthranilic oxidase was significantly higher ($P < 0.05$) in rats fed the indispensable amino acid mixture containing leucine than in rats fed the basal diet. In addition, the activity of this enzyme in rats fed the indispensable amino acid mixture without leucine (Group C) was significantly higher ($0.10 < P < 0.05$) than that obtained for rats fed the basal diet. The absence of leucine in the indispensable amino acid mixture resulted in a significantly lower activity ($0.10 < P < 0.05$) of 3-hydroxyanthanilic oxidase in the liver of rats.

The activity of this enzyme in the liver of rats fed each of the three diets was not consistent with results reported by Wallin (69). Wallin observed that the excretion of 3-hydroxyanthranilic acid in the urine of rats fed either of the indispensable amino acid mixtures was significantly higher ($P < 0.01$) than the excretion in rats fed the basal diet. When leucine was removed from the amino acid mixture, Wallin found that rats excreted significantly smaller ($P < 0.01$) amounts of 3-hydroxyanthranilic acid than in rats fed the complete amino acid mixture.

On the basis of her work, Wallin (69) suggested that leucine causes a block in the tryptophan to NAD pathway beyond the formation of the metabolite 3-hydroxyanthranilic acid. Therefore, one might expect lowered enzyme activity of 3-hydroxyanthranilic oxidase in the group of rats fed the amino acid mixture containing leucine (Group B), if this enzyme is affected by leucine. However, as observed in the present study, the activity of 3-hydroxyanthranilic oxidase in the liver of rats

fed the indispensable amino acid mixture containing leucine tended to be significantly higher than that for rats fed either the indispensable amino acid mixture without leucine or the basal diet. Therefore, one might conclude that leucine has a stimulatory effect on the activity of this enzyme in particular, rather than inhibiting its activity. It is possible that leucine adversely affects the activity of other enzymes beyond 3-hydroxyanthranilic oxidase in the pathway, resulting in a buildup of the metabolite 3-hydroxyanthranilic acid in the urine of rats fed a diet containing leucine.

Interrelationships Between the Activity of Kynurenine Hydroxylase, 3-Hydroxyknureninase, and 3-Hydroxyanthranilic Oxidase

As stated previously, the diet containing the complete mixture of amino acids caused no significant differences in the activity of kynurenine hydroxylase and 3-hydroxykynureninase and a significant increase in the activity of 3-hydroxyanthranilic oxidase as compared to the activity of these enzymes in rats fed the amino acid mixture without leucine or the basal diet. This finding indicates that leucine does not adversely affect the activity of the three enzymes being studied. In the present study, the leucine content of the complete indispensable amino acid mixture was only 0.3 percent leucine, which might indicate that the effect of dietary leucine cannot be seen at this level of leucine ingestion.

Because the activity of 3-hydroxykynureninase and 3-hydroxyanthranilic oxidase were higher in rats fed each of the other two diets, one might conclude that leucine has a stimulatory effect on the activity of each of these enzymes.

Although patterns of enzymatic lesions frequently correspond to changes in urinary tryptophan metabolites, these two parameters have not been studied simulataneously. Enzymatic activities represent the function of the particular organ in which they are studied, such as the liver, whereas variations in the urinary excretion of metabolites express the tryptophan metabolism function of the body as a whole (22). Further insight might be gained on the effect of leucine on the tryptophan to NAD pathway if both the urinary excretion of tryptophan metabolites and the activity of the various enzymes in the pathway are studied simultaneously in the same animal.

CHAPTER V

SUMMARY

The effect of adding a mixture of indispensable amino acids as found in 1.71 percent zein, with or without leucine, to a basal 6 percent casein, niacin-free diet on weight gain, feed consumption, and activity of the enzymes kynurenine hydroxylase, 3-hydroxykynureninase, and 3-hydroxyanthranilic oxidase in the rat have been studied. Twenty-seven young, male rats of the Sprague-Dawley-Long-Evans strain in three groups of nine rats each were fed one of the experimental diets for a two-week period. Livers from each rat were removed and divided into three sections for measurement of the activity of kynurenine hydroxylase, 3-hydroxykynureninase, and 3-hydroxyanthranilic oxidase. Spectrophotometric methods were used to determine the activity of each enzyme being studied.

There were no significant differences in weight gains and feed consumption between rats fed the basal diet and rats fed the basal diet supplemented with either of the two amino acid mixtures.

The values obtained for activity of kynurenine hydroxylase also were not significantly different between rats fed each of the three experimental diets.

In comparison with rats fed the basal diet, the activity of 3-hydroxykynureninase in the liver of rats fed the indispensable amino acid mixture without leucine approached significance ($P > 0.10$).

However, there were no significant differences in the activity of this enzyme between rats fed either of the two amino acid mixtures or between rats fed the indispensable amino acid mixture containing leucine and rats fed the basal diet.

The activity of 3-hydroxyanthanilic oxidase was significantly higher ($P < 0.05$) in rats fed the indispensable amino acid mixture containing leucine than in rats fed the basal diet. In addition, the activity of this enzyme in rats fed the indispensable amino acid mixture without leucine was significantly higher ($0.10 < P < 0.05$) than the value for the group of rats fed the basal diet. When leucine was removed from the indispensable amino acid mixture, the activity of 3-hydroxyanthranilic oxidase in the liver of rats fed this diet was significantly lower ($0.10 < P < 0.05$) than the value for the activity in rats fed the indispensable amino acid mixture containing leucine.

The diet containing the complete mixture of amino acids caused no significant differences in the activity of kynurenine hydroxylase and 3-hydroxykynureninase and a significant increase in the activity of 3-hydroxyanthranilic oxidase, as compared to the activity in rats fed the amino acid mixture without leucine or the basal diet. This finding might indicate that leucine does not adversely affect the activity of the three enzymes being studied. Because the activity of 3-hydroxykynureninase and 3-hydroxyanthranilic oxdiase was higher in rats fed each of the two amino acid mixtures than in rats fed the basal diet, one might conclude that leucine has a stimulatory effect on the activity of these two enzymes.

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